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Published in:
Annual Review of Microbiology

DOI:
10.1146/annurev.micro.53.1.315

Publication date:
1999

Annu. Rev. Microbiol. 1999. 53:315–51

BACTERIAL BIOCATALYSTS: Molecular Biology, Three-Dimensional Structures, and Biotechnological Applications of Lipases

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Key Words secretion, directed evolution, enantioselectivity

■ **Abstract** Bacteria produce and secrete lipases, which can catalyze both the hydrolysis and the synthesis of long-chain acylglycerols. These reactions usually proceed with high regioselectivity and enantioselectivity, and, therefore, lipases have become very important stereoselective biocatalysts used in organic chemistry. High-level production of these biocatalysts requires the understanding of the mechanisms underlying gene expression, folding, and secretion. Transcription of lipase genes may be regulated by quorum sensing and two-component systems; secretion can proceed either via the Sec-dependent general secretory pathway or via ABC transporters. In addition, some lipases need folding catalysts such as the lipase-specific foldases and disulfide-bond–forming proteins to achieve a secretion-competent conformation. Three-dimensional structures of bacterial lipases were solved to understand the catalytic mechanism of lipase reactions. Structural characteristics include an α/β hydrolase fold, a catalytic triad consisting of a nucleophilic serine located in a highly conserved Gly-X-Ser-X-Gly pentapeptide, and an aspartate or glutamate residue that is hydrogen bonded to a histidine. Four substrate binding pockets were identified for triglycerides: an oxyanion hole and three pockets accommodating the fatty acids bound at positions *sn*-1, *sn*-2, and *sn*-3. The differences in size and the hydrophilicity/hydrophobicity of these pockets determine the enantiopreference of a lipase. The understanding of structure-function relationships will enable researchers to tailor new lipases for biotechnological applications. At the same time, directed evolution in combination with appropriate screening

systems will be used extensively as a novel approach to develop lipases with high stability and enantioselectivity.

CONTENTS

The authors dedicate this article to Professor Dr. Ulrich K. Winkler on the occasion of his 70th birthday. Uli Winkler was among the first to isolate bacterial lipases from *Serratia marcescens* and *Pseudomonas aeruginosa* and to study their physiology and biochemical properties.

INTRODUCTION

Nearly 100 years ago, a landmark report was published by C Eijkmann (34) describing the following simple experiment: beef tallow spread on the ground of a glass plate was overlaid with agar that was inoculated with different bacteria. After 3–4 days of incubation, Ca, Na, and NH_4 soaps had formed. Eijkmann concluded that lipases had been produced and secreted by the bacteria, among them *Bacillus*

pyocyaneus (today named *Pseudomonas aeruginosa*), *Staphylococcus pyogenes aureus* (*S. aureus*), *B. prodigiosus* (today *Serratia marcescens*), and *B. fluorescens* (today *P. fluorescens*). *B. coli communis* (*Escherichia coli*) was found to be lipase negative (34). Only a few lipase-producing bacteria were further characterized (95), but research was intensified when it became generally accepted that lipases remain enzymatically active in organic solvents (166), making them ideal tools for the organic chemist. The aim of this review is not to discuss every lipase described in the literature but rather to present recent information on selected and novel lipases. It covers the period from 1994, when a comprehensive review on bacterial lipases appeared (58), until 1998. During this time a number of novel lipases were cloned and characterized, considerable progress was made in understanding the regulation of lipase gene expression, and detailed knowledge became available concerning folding and secretion. Moreover, three-dimensional structures of lipases were solved and used to explain functional characteristics. These developments, as well as other aspects such as the important role of lipases in biotechnological applications, are discussed, with special emphasis on enantioselective biotransformations. The enormous interest in lipases is reflected by a rapidly growing number of excellent review articles and monographs covering molecular biology, biochemical characterization, three-dimensional structures, and biotechnological applications of lipases from prokaryotic and eukaryotic origins (4, 46, 58, 59, 66, 102, 121, 122, 125, 165).

DEFINITION OF A LIPASE

What exactly is a lipase? At present, there is no satisfying answer to this simple question. Lipolytic reactions occur at the lipid-water interface where lipolytic substrates usually form an equilibrium between monomeric, micellar, and emulsified states. Until recently, two criteria have been used to classify a lipolytic enzyme as a "true" lipase (EC 3.1.1.3): (*a*) It should be activated by the presence of an interface, that is, its activity should sharply increase as soon as the triglyceride substrate forms an emulsion. This phenomenon was termed "interfacial activation" (124). (*b*) It should contain a "lid" (see below), which is a surface loop of the protein covering the active site of the enzyme and moving away on contact with the interface (17, 24, 158). However, these obviously suggestive criteria proved to be unsuitable for classification, mainly because a number of exceptions were described of enzymes having a lid but not exhibiting interfacial activation (159). Therefore, lipases are simply defined as carboxylesterases catalyzing the hydrolysis (and synthesis) of long-chain acylglycerols (37). There is no strict definition available for the term "long-chain," but glycerolesters with an acyl chain length of $\geq$10 carbon atoms can be regarded as lipase substrates, with trioleoylglycerol being the standard substrate. Hydrolysis of glycerolesters with an acyl chain length of $<$10 carbon atoms with tributyrylglycerol (tributyrin) as the standard substrate

usually indicates the presence of an esterase (62). It should be emphasized that most lipases are perfectly capable of hydrolyzing these esterase substrates.

SCREENING FOR LIPASE ACTIVITY

Hydrolysis

Microbiologists generally want to use a simple and reliable plate assay allowing the identification of lipase-producing bacteria. The most widely used substrates are tributyrin and triolein, which are emulsified mechanically in various growth media and poured into a petri dish. Lipase production is indicated by the formation of clear halos around the colonies grown on tributyrin-containing agar plates (6) and orange-red fluorescence visible on irradiation with a conventional UV hand lamp at $\lambda = 350$ nm on triolein plates, which additionally contain rhodamine B (74). Lipase activity in bacterial culture supernatants is determined by hydrolysis of *p*-nitrophenylesters of fatty acids with various chain lengths ($\geq$C-10) and spectrophotometric detection of *p*-nitrophenol at 410 nm. However, care must be taken to interpret the results because these fatty acid monoester substrates are also hydrolyzed by esterases. This problem can be overcome by using the triglyceride derivative 1,2-*O*-dilauryl-*rac*-glycero-3-glutaric acid resorufin ester (available from Boehringer Mannheim Roche GmbH, Germany), yielding resorufin, which can be determined spectrophotometrically at 572 nm or fluorometrically at 583 nm. A number of novel fluorogenic alkyldiacylglycerols were synthesized and used for analysis of both lipase activity and stereoselectivity (167). A more laborious but reliable method for identifying a "true" lipase is the determination of fatty acids liberated from a triglyceride, usually trioleoylglycerol, by titration (62). Automated equipment allows the parallel assay of a large number of samples. Determination of kinetics of lipolysis requires a tight control of the interfacial quality achieved by using the monolayer technique: A lipid film is spread at the air/water interface in a so-called "zero-order" trough consisting of a substrate reservoir and a reaction compartment. Lipase-catalyzed hydrolysis of the lipid monolayer results in changes of the surface pressure, which can be readjusted automatically by a computer-controlled barostat (111). It should be emphasized that this technique requires expensive equipment and experienced personnel.

Synthesis

Biotechnological applications of lipases prompt a demand for techniques to determine their activity and, if relevant, stereoselectivity. A standard reaction is the lipase-catalyzed esterification of an alcohol with a carboxylic acid, e.g. the formation of octyl laurate from lauric acid and *n*-octanol reacted in an organic solvent (114). The initial rate of ester formation can be determined by gas chromatography. No single method is available to determine the enantioselectivity of a lipase-catalyzed organic reaction. Generally, the enantioselectivity of product

formation is determined either by gas chromatography or high performance liquid chromatography (HPLC), with chirally modified columns. Basically, two types of enantioselective lipase-catalyzed reactions are possible: (*a*) desymmetrization of prochiral substrates in hydrolysis or acylation reactions and (*b*) kinetic resolution of racemic mixtures, hydrolysis or acylation again being the two options. Recently, a number of screening methods for lipases have been reviewed, including those which allow the conventional determination of enantioselectivity and regioselectivity (1). However, without modification they are not suitable for high throughput screening.

CLASSIFICATION OF BACTERIAL LIPASES

A search of available data banks (GenBank, Swiss Protein Sequence Database, The Protein Information Resource, The Protein Research Foundation Database, and The Brookhaven Protein Databank) revealed 217 entries of lipolytic enzymes from bacteria (search completed in November 1998). Many of the entries turned out to be redundant, and, finally, 47 different lipases were identified and grouped into six families based on amino acid sequence homology (Table 1) (JL Arpigny & K-E Jaeger, submitted for publication).

Family I comprises a total of 22 members subgrouped into 6 subfamilies. Subfamilies I.1 and I.2 extend the previously described *Pseudomonas* group I and II lipases encoded in an operon together with their cognate intramolecular chaperones, which have been designated Lif (lipase-specific foldases) (58). These lipases are secreted via the type II pathway, whereas those belonging to subfamily I.3 use the type I secretion pathway (see below). The *Bacillus* lipases grouped in subfamily I.4 are the smallest lipases known, with a molecular mass of 19.6 kDa. They seem to be well suited for biotechnological applications as is *B. thermocatenulatus* lipase belonging to subfamily I.5 (126). Lipases from *Staphylococcus hyicus* and *S. aureus* belong to the best characterized lipases originating from gram-positive bacteria (140). Family II has been described before as a novel family of lipolytic enzymes with unknown function (157). We have recently added to this family as a novel member an esterase located in the outer membrane of *P. aeruginosa* (S Wilhelm, J Tommassen, K-E Jaeger, submitted for publication). This enzyme is an autotransporter belonging to a previously identified family of channel-forming bacterial virulence factors (89). Determination of the three-dimensional structure for the *Streptomyces scabies* enzyme indicated that the tertiary fold of esterases belonging to this family may be substantially different from the α/β hydrolase fold found in most lipases. The members of family III contain extracellular lipases from *Streptomyces* spp. and the psychrophilic strain of *Moraxella* sp. Members of family IV belong to the group of the cold-adapted lipases exhibiting similarity to mammalian hormone-sensitive lipases (80). They contain the active-site serine residue in a consensus pentapeptide GDSAG, which is located close to the N terminus of the protein, and, in addition, they have another strictly conserved HGGG motif

TABLE 1 Families of lipolytic enzymes

Family	Sub-family	Enzyme-producing species	Accession no.	Similarity[b] (%)	Properties
I	1	*Pseudomonas aeruginosa*[a]	D50587	100	True lipases
		Pseudomonas fluorescens C9	AF031226	95	
		Vibrio cholerae	X16945	57	
		Acinetobacter calcoaceticus	X80800	43	
		Pseudomonas fragi	X14033	40	
		Pseudomonas wisconsinensis	U88907	39	
		Proteus vulgaris	U33845	38	
	2	*Burkholderia glumae*[a]	X70354	35	
		Chromobacterium viscosum[a]	Q05489	35	
		Burkholderia cepacia[a]	M58494	33	
		Pseudomonas luteola	AF050153	33	
	3	*Pseudomonas fluorescens* SIKW1	D11455	14	
		Serratia marcescens	D13253	15	
	4	*Bacillus subtilis*	M74010	16	
		Bacillus pumilus	A34992	13	
	5	*Bacillus stearothermophilus*	U78785	15	
		Bacillus thermocatenulatus	X95309	14	
		Staphylococcus hyicus	X02844	15	Phospholipase
		Staphylococcus aureus	M12715	14	
		Staphylococcus epidermidis	AF090142	13	
	6	*Propionibacterium acnes*	X99255	14	
		Streptomyces cinnamoneus	U80063	14	
II		*Pseudomonas aeruginosa*	AF005091	100	o.m.-bound esterase
		Aeromonas hydrophila	P10480	31	Acyltransferase
		Salmonella typhimurium	AF047014	17	o.m.-bound esterase
		Photorhabdus luminescens	P40601	17	
		Streptomyces scabies[a]	M57297	15	
III		*Streptomyces exfoliatus*[a]	M86351	100	Extracellular lipase
		Streptomyces albus	U03114	82	
		Moraxella sp.	X53053	33	Lipase 1

(*continued*)

Family	Sub-family	Enzyme-producing species	Accession no.	Similarity[b] (%)	Properties
IV		*Moraxella sp.*	X53868	100	Lipase 2
		Archaeoglobus fulgidus	AE000985	28	Carboxylesterase
		Alicyclobacillus acidocaldarius	X62835	25	
		Pseudomonas sp. *B11-1*	AF034088	24	
		Alcaligenes eutrophus	L36817	24	
		Escherichia coli	AE000153	20	Esterase
V		*Moraxella* sp.	X53869	100	Lipase 3
		Psychrobacter immobilis	X67712	88	
		Pseudomonas oleovorans	M58445	34	PHA-depolymerase
		Haemophilus influenzae	U32704	34	Putative esterase
		Sulfolobus acidocaldarius	AF071233	25	Esterase
		Acetobacter pasteurianus	AB013096	15	Esterase
VI		*Pseudomonas fluorescens*[a]	S79600	100	Esterases
		Synechocystis sp.	D90904	24	
		Spirulina platensis	S70419	22	
		Rickettsia prowazekii	Y11778	16	
		Chlamydia trachomatis	AE001287	15	

[a]Lipolytic enzymes with known 3-D structure.

[b]Similarities of amino acid sequences were determined with the program Megalign (DNAStar), with the first member of each family arbitrarily set at 100%.

of unknown function located immediately upstream of the active site consensus motif. It is interesting that families IV and V contain lipases belonging to either psychrophilic (*Moraxella* sp., *Pseudomonas* sp. B11-1, and *P. immobilis*) or thermophilic (*Archaeoglobus fulgidus* and *Sulfolobus acidocaldarius*) bacteria. The members of family V show structural similarities to dehalogenases, haloperoxidases, and epoxide hydrolases presumably exhibiting the α/β hydrolase fold as the characteristic tertiary structure. Family VI lists esterases that have partly been identified from genome sequences (*Synechocystis* sp., *Chlamydia trachomatis*). These esterases are small proteins presumably located in the bacterial cytoplasm with similarity to mammalian lysophospholipases.

Determination and comparison of specific activities and substrate specificities of different lipases are absolutely required to investigate the physiological function of lipases as well as to judge their usefulness for biotechnological applications.

However, serious doubts remain as to the comparability of results published by different laboratories. As outlined above, no standard substrate or assay system is available to determine specific lipase activities or to distinguish lipases from esterases. For this reason a recent study that compares the enzyme activities of 11 bacterial lipases obtained from both gram-positive and gram-negative bacteria is very important (141). All tested lipases showed high activity toward the short-chain triglyceride tributyrylglycerol and the long-chain trioctanoylglycerol, with lipases from *P. aeruginosa* and *Proteus vulgaris* being the most active. Phospholipids like 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine were either poor substrates (for lipases from *S. aureus, S. epidermidis, S. warneri, Acinetobacter calcoaceticus, Arthrobacter* sp., *P. aeruginosa, Burkholderia cepacia*, and *P. vulgaris*) or not hydrolyzed at all (by lipases from *B. thermocatenulatus* and *S. marcescens*). The only exception was *S. hyicus* lipase, which readily hydrolyzed phospholipids with high specific activity (141). In summary, it is obvious that many lipase genes are being found by accident and the corresponding proteins remain largely uncharacterized. Novel lipase-producing microorganisms have been described (10, 61, 149), but the cognate lipase genes and proteins still need to be isolated.

REGULATION OF LIPASE GENE EXPRESSION

Until recently, a significant number of prokaryotic lipase genes have been cloned, but the molecular mechanisms regulating their expression remain largely unknown. In *S. aureus* a global regulatory network was identified that coordinately controls the transcription of genes involved in pathogenesis. Two interacting genetic loci termed *agr* and *sar* were identified that activate the transcription of genes encoding extracellular proteins when the bacteria enter the stationary growth phase (50). Lipase gene expression seems to be regulated by the *agr* locus encoding a cell density-dependent regulatory system consisting of a two-component system with *agrC* as the signal transducer and *agrA* as the response regulator. A peptide pheromone encoded by *agrD* serves as the autoinducer. Because a mutation in *agrC* originally termed *xpr* was found to repress the extracellular lipase activity (142), it can be concluded that lipase expression in *S. aureus* is regulated by a quorum sensing system mediated through the two-component regulator *agrC/A* (92). Recently, the alternative sigma factor σ^B was shown to repress lipase synthesis; however, it remained unclear whether lipase gene expression or lipase protein secretion was affected (76).

A variety of *Streptomyces* strains produce extracellular lipase, a process that is dependent on the growth phase. Transcription of the gene *lipA* of *S. exfoliatus* M11 was dependent on the presence of the downstream gene *lipR* encoding a transcriptional activator that belongs to the LuxR family of bacterial regulators (132). This lipase-specific transcriptional regulator switches on lipase synthesis at the onset of the stationary growth phase, probably enabling *Streptomyces* to make use of triacylglycerol storage compounds (101, 132).

The insect pathogen *Xenorhabdus nematophilus* that exists in the intestine of the parasitic nematode *Steinernema carpocapsae* produces a lipase. Its biosynthesis is stimulated by *N*-β-hydroxybutanoyl homoserine lactone (HBHL), known as the autoinducer of the luminescent system of *Vibrio harveyi* (30). This finding suggests a quorum sensing type of lipase gene regulation that would involve, apart from the signal molecule HBHL, at least two genes encoding an autoinducer synthetase and a transcriptional activator, respectively.

A. calcoaceticus, a gram-negative soil bacterium, produces a number of lipolytic enzymes, among them an extracellular lipase encoded by *lipA* (72). The effects of various physiological factors on lipase gene expression were studied by using transcriptional *lipA*::*lacZ* fusions integrated into the *A. calcoaceticus* chromosome. Fatty acids produced by hydrolysis of the lipase substrate triolein strongly repressed expression of *lipA* (71). These findings were explained by proposing the existence of an as yet unidentified regulatory protein that is believed to repress lipase transcription on binding of a fatty acid (71).

In *P. aeruginosa*, transcription of the lipase operon *lipA/H* from promotor P1 requires the presence of the alternative sigma factor σ^{54} (60). Recently, primer extension analysis confirmed this result and also revealed the presence of a second promotor P2 located ~300 base pairs (bp) upstream of P1 (H Duefel, P Braun, W Quax, K-E Jaeger, manuscript in preparation). Because σ^{54}-dependent promotors require the presence of a cognate transcriptional activator (138), the existence of a lipase regulator *lipR* was proposed (60). Extracellular lipase is secreted when *P. aeruginosa* enters the stationary growth phase (145). This suggests a cell density-dependent regulation of lipase gene expression involving one of the quorum sensing systems identified in *P. aeruginosa* (150). Studies with *lipA*::*lacZ* fusions revealed that lipase gene expression is controlled via the *rhlR/I* (also named *vsmR/I*) system, including the autoinducer *N*-butyryl-homoserine lactone (BHL) (127), which controls the synthesis of *P. aeruginosa* rhamnolipid (97, 98) as well as several extracellular enzymes (15, 81). Characterization of a Tn*5* mutant exhibiting reduced lipase activity recently led to the identification of *lipR* encoding a transcriptional activator, which may be part of a two-component system. A second gene located upstream of *lipR*, which we tentatively name *lipQ*, encoding a putative signal transducing protein, was identified by searching the *Pseudomonas* genome project (www.pseudomonas.com/) (H Duefel, P Braun W Quax, K-E Jaeger, manuscript in preparation). In addition, the global regulator gene *gacA* was identified, which acts as part of another two-component system (119). Overproduction of GacA markedly increased extracellular lipase activity, presumably via activation of the *rhlR/I* system. Figure 1 summarizes the current status of the regulatory network controlling expression of the lipase operon *lipA/H* in *P. aeruginosa*.

In conclusion, a cell density-dependent regulation of extracellular lipase synthesis emerges as a general regulatory mechanism, especially in pathogenic bacteria with lipases qualifying as virulence factors. Additional activators or repressors of transcription responding to a variety of environmental signals seem to exist, making lipase gene expression a highly regulated system.

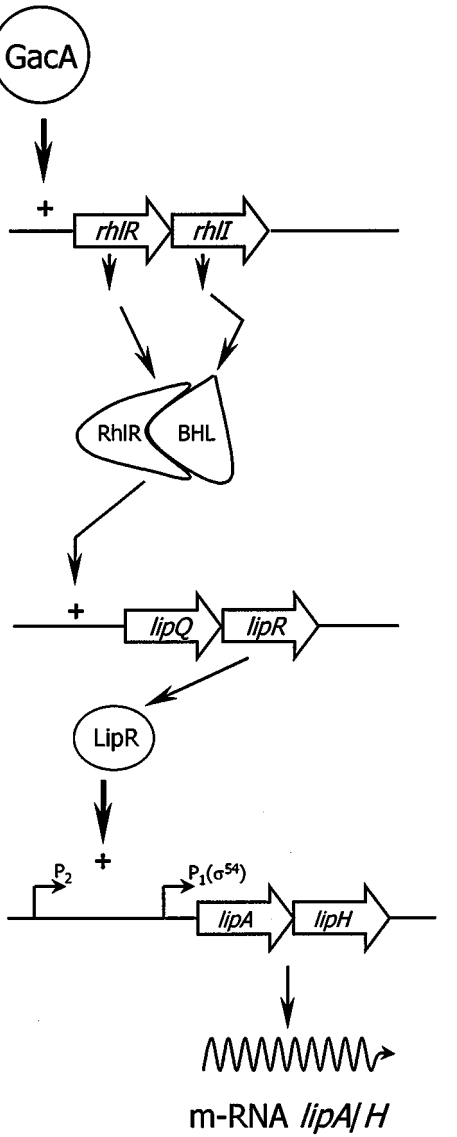

Figure 1 Model for the regulatory network controlling expression of the lipase operon *lipA/H* in *Pseudomonas aeruginosa* (see text for details). Stimulation of gene transcription is indicated by +.

SECRETION AND FOLDING

Lipases are extracellular enzymes, which must, therefore, be translocated through the bacterial membranes to reach their final destination. At present, three main secretion pathways have been identified (11, 107, 123), and it appears that lipases can use at least two of them. More recently, it became obvious that correct folding of lipases is needed to ensure proper secretion. Periplasmic folding catalysts include intramolecular Lif chaperones (58) and, at least in *P. aeruginosa*, accessory proteins involved in disulfide bond formation (A Urban, M Leipelt, T Eggert, K-E Jaeger, submitted for publication).

ABC Exporters

Lipases from *P. fluorescens* (32) and *S. marcescens* (3, 84) lack a typical N-terminal signal peptide, and they are secreted by an ABC exporter (also named type I secretion pathway) consisting of three different proteins (11). In *S. marcescens* the inner membrane protein LipB containing an ATP-binding cassette (ABC protein) confers the substrate specificity to the system. Additional components include LipC as a membrane fusion protein (MFP), which can be associated with both the inner and the outer membrane, and LipD as an outer membrane protein (OMP). Both lipase and metalloprotease are secreted through this ABC transporter (2); however, the cell-surface layer protein SlaA seems to be its natural substrate (64).

Secretion Across the Inner Membrane

Many lipases of both gram-positive and gram-negative bacteria possess an N-terminal signal sequence mediating their secretion through the inner membrane by means of the Sec translocase. In *E. coli* this is a multisubunit protein complex, consisting of the soluble dimeric SecA and a membrane-embedded complex formed by SecY, E, D, G, and F (31). A similar Sec-translocase exists in *Bacillus* species (139), which is presumably also involved in secretion of lipases.

Secretion Across the Outer Membrane

After being secreted through the inner membrane of gram-negative bacteria, lipases fold in the periplasm into an enzymatically active conformation. Subsequently, they are transported through the outer membrane by means of a complex machinery called the secreton, which consists of $\leq$14 different proteins forming the type II or general secretion pathway (107). In *P. aeruginosa*, lipase is secreted through such a secreton (38, 60), here encoded by 12 *xcp* genes organized in two divergently transcribed operons (38, 153). The Xcp proteins are located both in the inner and the outer membrane, with XcpQ forming a multimeric pore with a diameter of 95 Å (12). Recently, a similar secreton involved in secretion of *P. alcaligenes*

lipase was identified (43). When additional copies of the secreton were expressed from a cosmid introduced into *P. alcaligenes*, the production of extracellular lipase increased significantly (43). This effect, which has also been observed for *P. aeruginosa* lipase, suggests that the number of secreton complexes present in wild-type strains is probably low, thereby posing restrictions on the production of extracellular enzymes. Although similar multicomponent secretons have been identified in lipase-producing *A. hydrophila*, *X. campestris*, and *V. cholerae* (9, 104), their involvement in lipase secretion still has to be demonstrated.

Folding Catalysts

Specific intramolecular chaperones designated Lif proteins are required for folding of some lipases in the periplasm (58). These foldase proteins are usually encoded in an operon with their cognate lipases and have been identified in *P. aeruginosa* (18, 56, 57, 103, 164), *P. wisconsinensis* (M Hazbon, H Duefel, P Cornelis, K-E Jaeger, manuscript in preparation), *B. cepacia* (63), *B. glumae* (40, 41), *A. calcoaceticus* (73), and *V. cholerae* (99). The best studied Lif proteins are those from *P. aeruginosa* and *B. glumae*, which contain a hydrophobic N-terminal segment anchoring them to the inner membrane (40, 127). Truncated Lif proteins lacking this membrane anchor segment are still able to catalyze folding of lipases (133; F Rosenau, M Eller, K-E Jaeger, unpublished data), indicating that the membrane anchor domain may function to prevent secretion of Lif proteins. Lipase-Lif complexes have been immunoprecipitated with either antilipase or anti-Lif antisera (54, 55). The lipase:Lif ratio is as yet unknown; chemical cross-linking suggested a 1:1 complex, with Ca^{2+} ions needed for complex formation (134), whereas a ratio of 1:4 was suggested from in vitro refolding experiments (55). The interaction of lipase with Lif is specific, as shown by the inability of Lifs to activate heterologous lipases. The only known exception is the lipase from *P. alcaligenes*, which can partly be activated when coexpressed in *E. coli* together with *P. aeruginosa* Lif (35). Domain swapping revealed that an 138-amino-acid C-terminal domain of *B. glumae* Lif determines its substrate specificity (35). In an elegant study, *P. aeruginosa* Lif was randomly mutated by error-prone polymerase chain reaction (PCR) and coexpressed with the lipase gene in *E. coli*. Analysis of nonfunctional Lif mutant proteins allowed identification of residues Tyr-99 and Arg-115 as being essential for the interaction with lipase (135). Interestingly, a number of strictly conserved amino acid residues were identified in various Lif proteins (58), one of which is Tyr-99 in *P. aeruginosa* Lif (135).

Lipases produced by the gram-positive bacteria *S. aureus*, *S. epidermidis*, and *S. hyicus* are synthesized as pre-proenzymes, with an N-terminal pro-region of about 260 amino acids acting as a folding catalyst (45). The pro-region not only facilitates translocation of these lipases through the cytoplasmic membrane but also protects them from proteolytic attack. For *S. hyicus* lipase, removal of the pro peptide by an extracellular metalloprotease generates the 46-kDa mature lipase (7).

This pro-region is also capable of binding to heterologous proteins and protecting them from proteolytic degradation (86). When the pre-pro part of *S. hyicus* lipase was fused to *E. coli* OmpA, efficient secretion was observed not only in *S. hyicus* but also in *B. subtilis* (93).

Intramolecular chaperones have been studied extensively in various proteases, where they are found as short N-terminally located pro-peptides (137), which functionally resemble the pro-peptides found in lipases from gram-positive bacteria. They can be distinguished from Lif proteins, which are (*a*) not covalently attached to the N terminus of the protein and (*b*) anchored in the cytoplasmic membrane, thereby rendering them reusable. The main function of these chaperone proteins is to help the lipases in overcoming a kinetic barrier along their folding pathway. In addition, they may also function as temporary competitive inhibitors of their cognate enzymes.

Exported proteins including lipases often contain disulfide bonds, which are formed in the periplasm. In *E. coli* a complex system consisting of Dsb proteins mediates disulfide bond formation (108). DsbA is a thiol:disulfide oxidoreductase that oxidizes the Cys-SH residues to form a disulfide bond; DsbC is a thiol:disulfide isomerase that isomerizes wrongly oxidized disulfide bonds (94). The involvement of DsbA and DsbC in folding and secretion of lipase has been studied in *P. aeruginosa* (A Urban, M Leipelt, T Eggert, K-E Jaeger, submitted for publication). This lipase contains a single disulfide bond. In a *dsbA* mutant of *P. aeruginosa*, extracellular lipase production was reduced to 10% of the wild-type level as demonstrated by enzyme activity assays and Western blotting. In a *dsbA dsbC* double mutant, a residual lipase activity of only 1% of the wild-type activity was detected. Interestingly, a *P. aeruginosa dsbC* mutant exhibited a twofold increase in extracellular lipase activity (A Urban, M Leipelt, T Eggert, K-E Jaeger, submitted for publication). *A. hydrophila* lipase is secreted via the type II secretion pathway and also contains one disulfide bond (16). Exchange of both cysteine residues to serine did not reduce the enzymatic activity or prevent secretion; however, it rendered this lipase more sensitive to urea denaturation and proteolytic degradation (16). Similar results were obtained with *P. aeruginosa* lipase (K Liebeton, A Zacharias, K-E Jaeger, submitted for publication). Both Cys-183Ser and Cys-235Ser mutants still exhibited residual lipase activity, as did a Cys-183Ser/Cys-235Ser double mutant. Refolding to enzymatic activity of mutant lipases expressed in *E. coli* was not significantly impaired, indicating an important role of the disulfide bond for lipase stabilization in a secretion-competent rather than an enzymatically active form.

Figure 2 summarizes present knowledge on the mechanism of lipase secretion via the type II secretion pathway in *P. aeruginosa*. Important questions remain to be answered: (*a*) Does a specific secretion signal exist in lipases? (*b*) Which factors determine the specificity of the interaction between lipase and its cognate Lif protein? (*c*) Is lipase secreted through the Xcp complex separately or as a

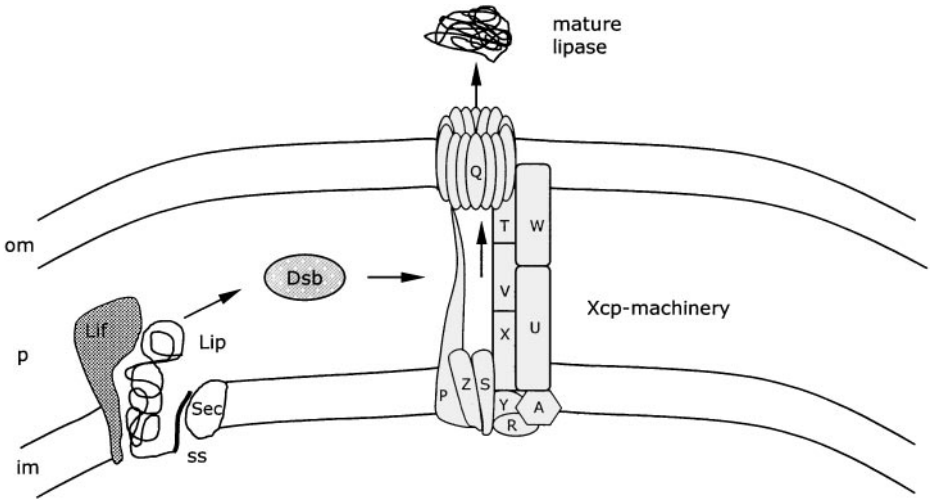

Figure 2 Model for the *Pseudomonas aeruginosa* lipase secretion pathway. Lipase (Lip) is secreted as prelipase including a signal sequence (ss) through the bacterial inner membrane (im) by a Sec-dependent mechanism. Interaction with the lipase-specific foldase Lif and Dsb proteins assists folding and formation of disulfide bonds in the periplasm (p). The final secretion through the outer membrane (om) is mediated by the Xcp machinery.

complex with Lif? (*d*) What is the mechanism leading to the close association with lipopolysaccharide observed for lipases isolated from bacterial culture supernatants (145)?

THREE-DIMENSIONAL STRUCTURES OF BACTERIAL LIPASES

Knowledge of their three-dimensional structures and the factors that determine their regiospecificity and enantiospecificity are traditionally essential to tailor lipases for specific applications. Human pancreatic lipase (163) and the lipase from the fungus *Rhizomucor miehei* (14, 28) were the first X-ray structures of lipases elucidated. Various other lipase structures of fungal origin followed, from *Geotrichum candidum* (130), *Fusarium solani* (91), *Candida rugosa* (48), *Candida antarctica* (155), *Humicola lanuginosa* (26), and *Rhizopus delemar* (26). In contrast, X-ray structures of bacterial lipases came only slowly. The first one, from *B. glumae*, appeared in 1993 (96). Several years later, it was followed by the lipases from *Chromobacterium viscosum* (77) [a lipase that appeared to be identical to the *B. glumae* lipase (151)] and *B. cepacia* (69, 79, 129). Recently, the X-ray structures of a lipase from *Streptomyces exfoliatus* (162), *Streptomyces scabies* (161) and an esterase from *Pseudomonas fluorescens* were published. The latter enzyme can

hydrolyze triacetin and tributyrin but cannot hydrolyze triglycerides of longer-chain fatty acids (68).

The Fold of Lipases

When the first lipase structures became known, it already appeared that they had very similar folds despite a lack of sequence similarity (22, 27, 143). A more extensive comparison with X-ray structures of other enzymes as diverse as haloalkane dehalogenase (39), acetylcholinesterase (147), dienelactone hydrolase (106), and serine carboxypeptidase (85) revealed that these enzymes all share the same folding pattern. Because they all catalyze a hydrolysis reaction, the common folding pattern was named the α/β hydrolase fold (100).

The canonical α/β hydrolase fold consists of a central, mostly parallel β sheet of eight strands with the second strand antiparallel (Figure 3). The parallel strands $\beta 3$ to $\beta 8$ are connected by α helices, which pack on either side of the central β sheet. The β sheet has a left-handed superhelical twist such that the surface of the sheet covers about half a cylinder and the first and last strands cross each other at an angle of $\sim 90^\circ$. The curvature of the β sheet may differ significantly among the various enzymes, and also, the spatial positions of topologically equivalent α helices may vary considerably. Excursions of the peptide chain at the C-terminal ends of strands

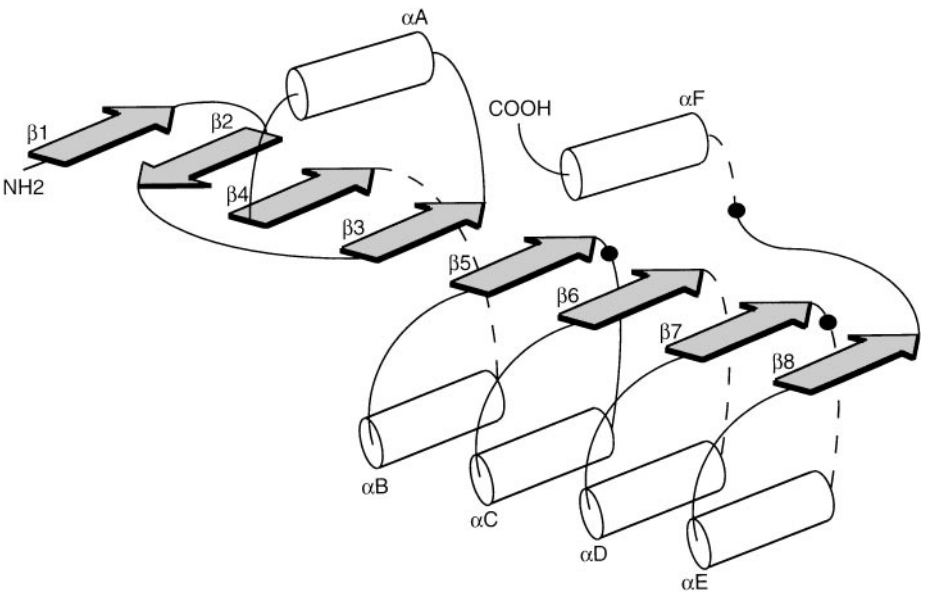

Figure 3 Canonical fold of α/β hydrolases (100). α Helices are indicated by *cylinders*, and β strands are indicated by *shaded arrows*. The topological position of the active-site residues is shown by a *solid circle*; the nucleophile is the residue after β strand 5, the Asp/Glu residue is after β strand 7, and the histidine residue is in the loop between $\beta 8$ and αF.

in the C-terminal half of the β sheet form the binding subdomains of the α/β hydrolase fold proteins. They differ substantially in length and architecture, in agreement with the large substrate diversity of these enzymes.

A detailed overview of the α/β hydrolase fold in lipases has been given by Schrag & Cygler (128). The bacterial lipase structures known so far obey the α/β hydrolase fold with some variations. The lipases from *B. glumae*, *B. cepacia*, and *C. viscosum* have six parallel β strands in the central β sheet of the α/β hydrolase fold [β3 to β8 (Figure 3)] (69, 77, 96). The *P. fluorescens* carboxylesterase contains seven strands, corresponding to strands β2 to β8 of the canonical fold (68), and the *S. exfoliatus* lipase has the full canonical α/β hydrolase fold with one extra antiparallel β strand added as strand 9. However, greater variation may be expected, for instance, for the smaller lipases from *Bacillus* (110) and the thioesterase from *E. coli* (87) and the higher-molecular-mass lipases from *Staphylococcus* species (109).

Recently, the X-ray structure of *P. aeruginosa* lipase was determined (DA Lang, K-E Jaeger, BW Dijkstra, in preparation). In contrast to the *B. glumae* and *B. cepacia* lipases, which belong to family I.2 lipases (58), the *P. aeruginosa* is a family I.1 lipase. Lipases of families I.1 and I.2 show ~60% amino acid sequence homology to each other [44% identity (148)]. Family I.1 lipases are somewhat smaller (about 285 amino acid residues instead of 320). *Pseudomonas* lipases are widely used in industry, especially for the production of chiral chemicals, which serve as basic building blocks in the synthesis of pharmaceuticals, pesticides, and insecticides (152). These enzymes show distinct differences in regioselectivity and enantioselectivity (120), despite a high amino acid sequence homology. For instance, Rogalska et al (120) found that the lipase from *C. viscosum* [which is identical to the *B. glumae* lipase (151)] reacts unspecifically [*sn*-3 (*R*)/*sn*-1 (*S*)] with trioctanoin, whereas the lipases from *B. cepacia* and *P. aeruginosa* (58) are absolutely specific for *sn*-1 fatty acid chains of natural substrates. The X-ray structure of *P. aeruginosa* shows the conserved α/β hydrolase folding pattern. Compared with the family I.2 lipases, the C-terminal antiparallel β sheet [residues 214 to 228 (96)] is missing, revealing more compact packing of the molecule. The active site is quite likely solvent accessible (Figure 4; see color insert).

The Catalytic Residues of Lipases

The active site of the α/β hydrolase fold enzymes consists of three catalytic residues: a nucleophilic residue (serine, cysteine, or aspartate), a catalytic acid residue (aspartate or glutamate), and a histidine residue, always in this order in the amino acid sequence (100). This order is different from that observed in any of the other proteins that contain catalytic triads. In lipases the nucleophile has so far always been found to be a serine residue, whereas the catalytic acid is either an aspartate or a glutamate residue.

The nucleophilic serine residue is located in a highly conserved Gly-X-Ser-X-Gly pentapeptide (100), which forms a sharp, γ-like turn between β5 of the canonical α/β hydrolase central β sheet and the following α helix. The γ turn is

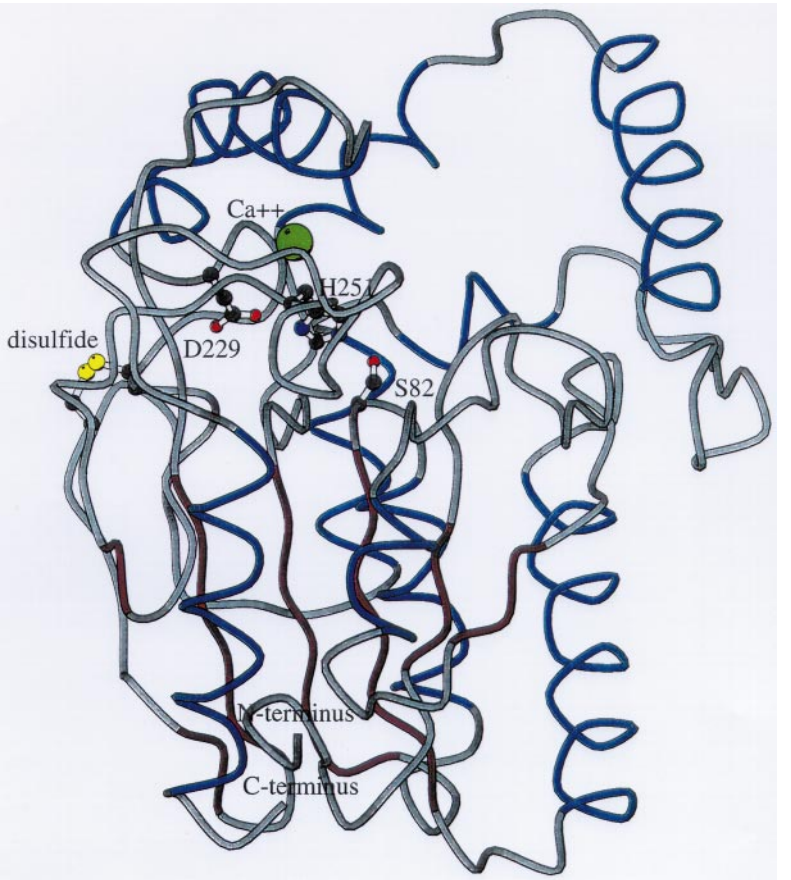

Figure 4 X-ray structure of *Pseudomonas aeruginosa* lipase. Indicated are the N- and the C- terminus, and the catalytic triad formed by the nucleophile (S82), the acid (D229) and the histidine residue (H251). In addition, the bound Ca^{++}-ion and the disulfide bond formed between residues C183 and C235 are shown.

characterized by energetically unfavorable main chain ϕ and φ torsion angles of the nucleophile. $\beta 5$, the γ turn and the following α helix form the most conserved structural feature of the α/β hydrolase fold. Because close contacts exist between the residues, two positions before and two positions behind the nucleophile, one and usually both residues at these positions are glycines. They are occasionally substituted for other small residues such as alanine, valine, serine, or threonine (23, 39, 82, 156). The strand-nucleophile-helix arrangement has been named the "nucleophile elbow." It positions the nucleophilic residue free of the active site surface and allows easy access on one side by the active site histidine residue and on the other by the substrate. The sharp turn also optimally positions the nucleophile at the N-terminal end of the following helix, thereby helping to stabilize the tetrahedral intermediate and the ionized form of the nucleophile.

In the prototypic α/β hydrolase fold, the catalytic acid (Asp or Glu) occurs in a reverse turn after strand 7 of the central β sheet (100). It is hydrogen-bonded to the active site histidine. However, the topological position of the catalytic acid seems to be variable: in pancreatic lipase it is positioned after strand 6 (163). This observation prompted Schrag et al (131) to redesign the active site of *G. candidum* lipase by shifting the position of the catalytic acid from strand 7 to strand 6. The double mutant retained ~10% of the wild-type activity, confirming the variability of the position of the catalytic acid. Similarly, the functionality of the catalytic acid in dehalogenase (Asp-260 positioned after strand 7) could be rescued by an Asn-148Asp mutation, a residue which is located after strand 6 (75). The X-ray structure of *B. glumae* lipase revealed Asp-263 as the catalytic acid residue that is hydrogen bonded to the catalytic histidine (96). Its position is not at the end of $\beta 7$, but there are several secondary structure elements lying between the end of $\beta 7$ and Asp-263. Site-directed mutagenesis experiments showed, however, only a modest reduction in activity of an Asp-263Ala mutant (42). Because the carboxyl group of Glu-287, which is located after strand 8, is close to the catalytic histidine, it is conceivable that Glu-287 takes over the role of Asp-263, either directly or via a water molecule (96).

The third catalytic residue in lipases is the catalytic histidine. This residue is located in a loop after β strand 8 of the α/β hydrolase fold. The length and conformation of this loop are variable (100).

The Catalytic Mechanism

Lipases are hydrolases acting on the carboxyl ester bonds present in acylglycerols to liberate fatty acids and glycerol. As detailed above, their active site consists of a Ser-His-Asp/Glu catalytic triad. This catalytic triad is similar to that observed in serine proteases, and therefore catalysis by lipases is thought to proceed along a similar path as in serine proteases. Hydrolysis of the substrate takes place in two steps (Figure 5).

It starts with an attack by the oxygen atom of the hydroxyl group of the nucleophilic serine residue on the activated carbonyl carbon of the susceptible lipid ester

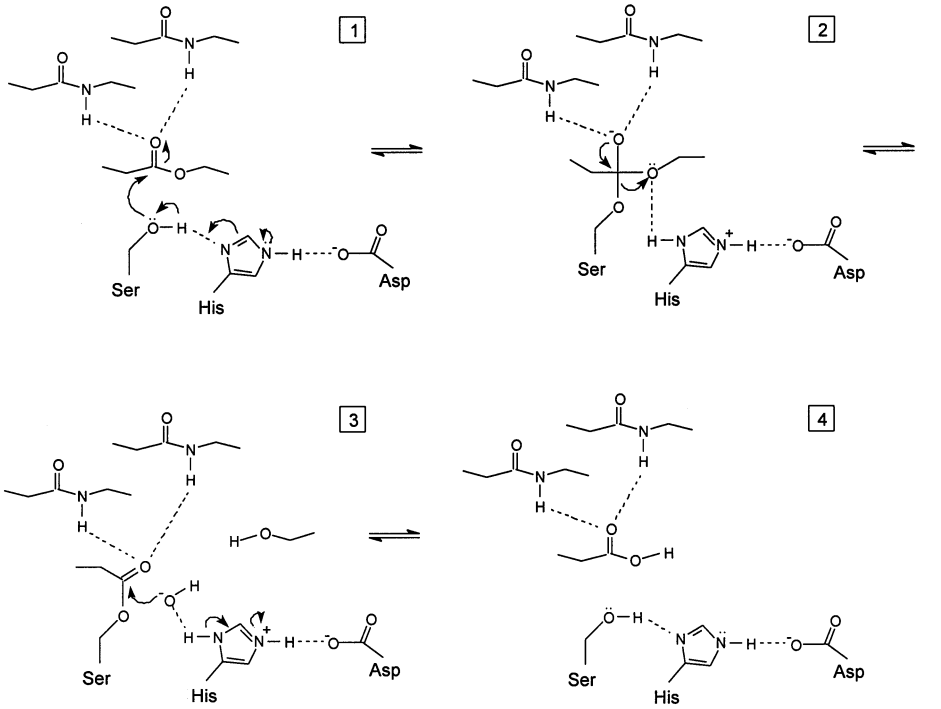

Figure 5 Reaction mechanism of lipases. [1] Binding of lipid, activation of nucleophilic serine residue by neighboring histidine and nucleophilic attack of the substrate's carbonyl carbon atom by Ser O^-. [2] Transient tetrahedral intermediate, with O^- stabilized by interactions with two peptide NH groups. The histidine donates a proton to the leaving alcohol component of the substrate. [3] The covalent intermediate ("acyl enzyme"), in which the acid component of the substrate is esterified to the enzyme's serine residue. The incoming water molecule is activated by the neighboring histidine residue, and the resulting hydroxyl ion performs a nucleophilic attack on the carbonyl carbon atom of the covalent intermediate. [4] The histidine residue donates a proton to the oxygen atom of the active serine residue, the ester bond between serine and acyl component is broken, and the acyl product is released.

bond (Figure 5). A transient tetrahedral intermediate is formed, which is characterized by a negative charge on the carbonyl oxygen atom of the scissile ester bond and four atoms bonded to the carbonyl carbon atom arranged as a tetrahedron (Figure 5). The intermediate is stabilized by the helix macrodipole of helix C (see Figure 3), and hydrogen bonds between the negatively charged carbonyl oxygen atom (the "oxyanion") and at least two main-chain NH groups (the "oxyanion hole"). One of the NH groups is from the residue just behind the nucleophilic serine; the other one is from the residue at the end of strand $\beta 3$ (65, 79, 129). The nucleophilicity of the attacking serine is enhanced by the catalytic histidine, to which a proton

from the serine hydroxyl group is transferred. This proton transfer is facilitated by the presence of the catalytic acid, which precisely orients the imidazole ring of the histidine and partly neutralizes the charge that develops on it. Subsequently, the proton is donated to the ester oxygen of the susceptible bond, which thus is cleaved. At this stage the acid component of the substrate is esterified to the nucleophilic serine (the "covalent intermediate"), whereas the alcohol component diffuses away (Figure 5). The next stage is the deacylation step, in which a water molecule hydrolyzes the covalent intermediate. The active-site histidine activates this water molecule by drawing a proton from it. The resulting OH^- ion attacks the carbonyl carbon atom of the acyl group covalently attached to the serine (Figure 5). Again, a transient negatively charged tetrahedral intermediate is formed, which is stabilized by interactions with the oxyanion hole. The histidine donates a proton to the oxygen atom of the active serine residue, which then releases the acyl component. After diffusion of the acyl product the enzyme is ready for another round of catalysis (Figure 5).

Evidence for this mechanism has come from various studies, particularly inhibitor binding to lipases and their structural analysis (25, 33, 47, 79, 88). In addition, a crystallographic analysis of the reaction catalyzed by haloalkane dehalogenase, another α/β hydrolase enzyme, provided definitive evidence for the occurrence of a covalent intermediate (160).

Interfacial Activation

Lipolytic enzymes are characterized by their drastically increased activity when acting at the lipid-water interface of micellar or emulsified substrates (124), a phenomenon called interfacial activation. This increase in enzymatic activity is triggered by structural rearrangements of the lipase active-site region, as witnessed from crystal structures of lipases complexed by small transition state analogs (17, 24, 158). In the absence of lipid-water interfaces, the active site is covered by a so-called "lid." However, in the presence of hydrophobic substances, the lid is opened, making the catalytic residues accessible to substrate and exposing a large hydrophobic surface. This hydrophobic surface is presumed to interact with the lipid interface. The lid may consist of a single helix (17, 24), or two helices (69, 129), or a loop region (49). However, not all lipases show this interfacial activation. Notable exceptions are the 19-kDa lipase from *B. subtilis* (83), cutinase (91), and guinea pig pancreatic lipase (53). These lipases lack a lid that covers the active site in the absence of lipid-water interfaces.

Substrate Binding

Extensive research has been carried out to identify the binding regions of the acyl and alcohol portions of the substrate in the various lipases and to rationalize the observed enantioselectivity. The X-ray structures of *R. miehei* lipase complexed with a C6 phosphonate inhibitor (25), of *C. rugosa* lipase with a long sulfonyl chain (49), of the human pancreatic lipase/colipase complex covalently

inhibited by the two enantiomers of a C11 alkyl chain phosphonate (33), and of porcine pancreatic lipase covalently inhibited by ethylene glycol monooctylether (51) represent important steps in mimicking the natural tetrahedral intermediates. However, none of those compounds resembled a true triglyceride. A first structural view of lipase stereoselectivity toward secondary alcohols was obtained by Cygler et al (21), who succeeded in complexing (*R*)- and (*S*)-methyl ester hexylphosphonate transition state analog to *C. rugosa* lipase. In the fast-reacting, (*R*)-enantiomer, a hydrogen bond is present between the alcohol oxygen of the substrate and the NE2 atom of the active site histidine. This hydrogen bond is absent in the slow-reacting, (*S*)-enantiomer, and therefore it was suggested that this hydrogen bond is responsible for the stereospecificity of the enzyme. Uppenberg et al (156) obtained similar results with *C. antarctica* lipase B complexed with a long-chain polyoxyethylene detergent. However, molecular dynamics calculations by Uppenberg et al suggest that the hydrogen bond is not only present in the fast-reacting (*R*)-enantiomer but also in the slow reacting (*S*)-enantiomer. Therefore, they concluded that the enzyme's enantioselectivity cannot simply be explained by the presence or absence of this hydrogen bond but that other factors such as the size of the alcohol-binding pocket may play a role as well. Longhi et al (88) prepared a complex of cutinase with an enantiopure triglyceride analog with three C4 alkyl chains, R_C-(R_P,S_P)-1,2-dibutylcarbamoylglycero-3-*O*-*p*-nitrophenyl butylphosphonate (90). Although this inhibitor was expected to reveal the stereospecific substrate interactions with the protein, it unfortunately was bound in the active site in an exposed position, with the alkyl chains not interacting with any amino acid residues of the enzyme.

A breakthrough came with the work of Lang et al (78, 79), who determined the X-ray structure of *B. cepacia* lipase in complex with an analog of medium alkyl chain length, R_C-(R_P,S_P)-1,2-dioctylcarbamoyl-glycero-3-*O*-*p*-nitrophenyl octylphosphonate (TC8). The enzyme is in the open conformation with the lid displaced to allow access of the substrate analog. Density for the complete inhibitor is visible, allowing an unambiguous definition of the substrate-binding mode. Four binding pockets were detected: an oxyanion hole and three pockets that accommodate the *sn*-1, *sn*-2, and *sn*-3 fatty acid chains (Figure 6). The boomerang-shaped active site (69, 129) is divided into a large hydrophobic groove, in which the *sn*-3 acyl chain snugly fits, and a part that embeds the inhibitor's alcohol moiety. The alcohol-binding pocket can be subdivided into a mixed hydrophilic/hydrophobic cleft for the *sn*-2 moiety of the substrate and a smaller hydrophobic groove for the *sn*-1 chain. Van der Waals interactions are the main forces that keep the radyl groups of the triglyceride analog in position. In addition, a hydrogen bond between the ester oxygen atom of the *sn*-2 chain and the NE2 atom of the active site histidine contributes to fix the position of the inhibitor. This hydrogen bond is equivalent to the one observed by Cygler et al (21) in *C. rugosa* lipase.

The bound lipid analog assumes the bent-tuning-fork conformation preferred by lipids at an interface (105). Because the *sn*-2 pocket provides the most intimate interactions with the substrate, this pocket is presumably the one that predominantly determines the enzyme's stereopreferences.

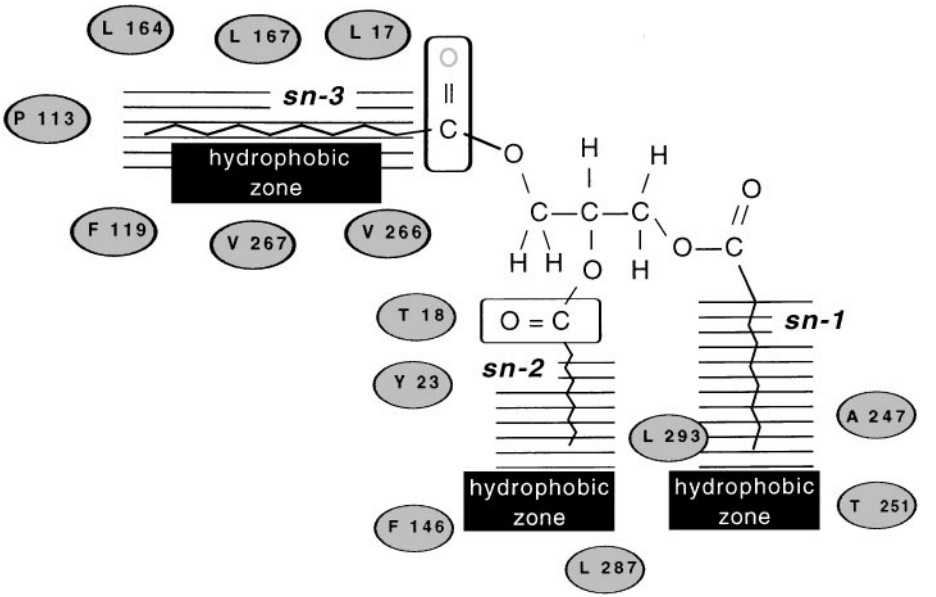

Figure 6 Active site of *Burkholderia cepacia* lipase. The binding pockets for the *sn*-1, *sn*-2, and *sn*-3 moieties of the lipid substrate are indicated. Also indicated are the residues lining these binding pockets.

Structural Determinants of Enantiomeric Selectivity

B. cepacia lipase is one of the most widely used enzymes for the enantiomeric resolution of esters of secondary alcohols (152). It has a preference for R_C over S_C compounds (79), but both enantiomers can be converted, demonstrating that the less preferred enantiomer can also be productively bound in the active site. Modeling of an S_C-TC8 compound in the active site of *B. cepacia* lipase revealed that the acyl chain bound to the primary hydroxyl group of the glycerol moiety clashes with the hydrophobic side chains of Leu-287 and Ile-290 (78, 79). To prevent this unfavorable interaction, either the amino acid side chains have to be moved out of the way or the substrate should undergo a conformational change, for instance, via a rotation about a single C-C bond. Hirose et al (52) have carried out site-directed mutagenesis experiments to probe the importance of various amino acid residues for the stereoselectivity of *B. cepacia* lipase. They succeeded in changing the enzyme's enantioselectivity from an R_C to S_C specificity by introducing a combination of three mutations, Val-266Leu, Leu-287Ile, and Phe-221Leu. Val-266 is located at the entrance of the acyl pocket (*sn*-3 pocket), whereas Leu-287 is at the beginning of the *sn*-2 pocket. Phe-221 is at the surface of the enzyme, $\sim$20 Å away from the inhibitor. Whereas the Leu-287Ile and Val-266Leu substitutions can be envisaged to affect the size and width of the *sn*-2 and *sn*-3 pockets, respectively, Phe-221 seems too far away to influence directly the enzyme's

stereospecificity. Nevertheless, the Phe-221Leu mutation on its own was reported to decrease slightly the enzyme's enantioselectivity (52). Clearly, further research is required to elucidate the role of Phe-221 as an enantioselectivity-determining factor.

In the past, it has been found that *B. cepacia* lipase shows the largest enantioselectivity if one of the substituents differs significantly in size from the other (67). This observation can now be rationalized; the enzyme contains a large hydrophobic groove in which the *sn*-3 acyl chain fits, a mixed hydrophilic/hydrophobic cleft for the *sn*-2 moiety of the substrate, and a smaller hydrophobic groove for the *sn*-1 chain. The differences in size and the hydrophilicity/hydrophobicity of the various pockets determine the enzyme's enantiopreferences and regiopreferences.

BIOTECHNOLOGICAL APPLICATIONS OF LIPASES

The commercial use of lipases is a billion-dollar business that comprises a wide variety of different applications. In the area of detergents, about 1000 tons of lipases are sold every year (44). Lipases also play an important role in the production of food ingredients (59). An example is the *Rhizomucor miehei* lipase-catalyzed transesterification reaction replacing palmitic acid by stearic acid to provide the stearic-oleic-stearic triglyceride with the desired melting point for use in chocolate (cocoa butter substitute) (19). Other applications of increasing interest include use of lipases in removing the pitch from pulp in the paper industry (36), in flavor development for dairy products and beverages, and in synthetic organic chemistry (13, 29, 35a, 59, 125, 144, 152).

Lipases as Catalysts in Synthetic Organic Chemistry

The number of reports concerning the use of lipases as catalysts in synthetic organic chemistry is increasing considerably (13, 29, 35, 125, 144). In addition to regioselective hydrolysis, acylation, or transesterification, which includes protective and deprotective methodologies, an incredibly wide variety of enantioselective processes have been reported. As outlined above, the two types of enantioselective organic transformations catalyzed by lipases are reactions of prochiral substrates and the kinetic resolution of racemates. Originally, prochiral or chiral alcohols or carboxylic acid esters served as the two main classes of compounds. However, during the last dozen years the scope has been extended to include cyanohydrins, chlorohydrins, diols, α- and β-hydroxy acids, amines, diamines, and amino alcohols. In principle, the most important classes of organic compounds can thus be produced enantioselectively by using lipase-catalysis. Either an aqueous medium is chosen (hydrolysis reactions), or organic solvents (70) (acylation or transesterification) are used. Typical catalysts for these synthetic organic reactions are bacterial lipases from *P. aeruginosa*, *P. fluorescens*, and other *Pseudomonas* species, *B. cepacia*, *C. viscosum*, *B. subtilis*, *Achromobacter* sp., *Alcaligenes* sp., and

S. marcescens. In view of the large number of publications from academic institutions concerning enantioselective reactions, it may be surprising that only a few cases of industrial processes are known. An example of considerable economic importance concerns the production of the calcium antagonist Diltiazem, a major pharmaceutical used in the treatment of high blood pressure. The key step is the kinetic resolution of a racemic mixture of a chiral epoxy ester based on the preferential hydrolysis of one enantiomeric form (125, 136) (Figure 7). Specifically, the bacterial lipase from *S. marcescens* catalyzes the hydrolysis of the (2*S*,3*R*)-configurated methyl *p*-methoxyphenylglycidate, which is easily separated from the desired (2*R*,3*S*) ester. At maximum theoretical conversion (∼50%), the enantiomeric excess (ee) of (2*R*,3*S*)-methyl-*p*-methoxyphenylglycidate is >98%. Since 1993, Tanabe manufactured more than 50 tons of this chiral building block per year, which is then converted into Diltiazem.

Another notable example of the industrial application of a lipase in enantioselective organic chemistry concerns the BASF AG process for preparing optically

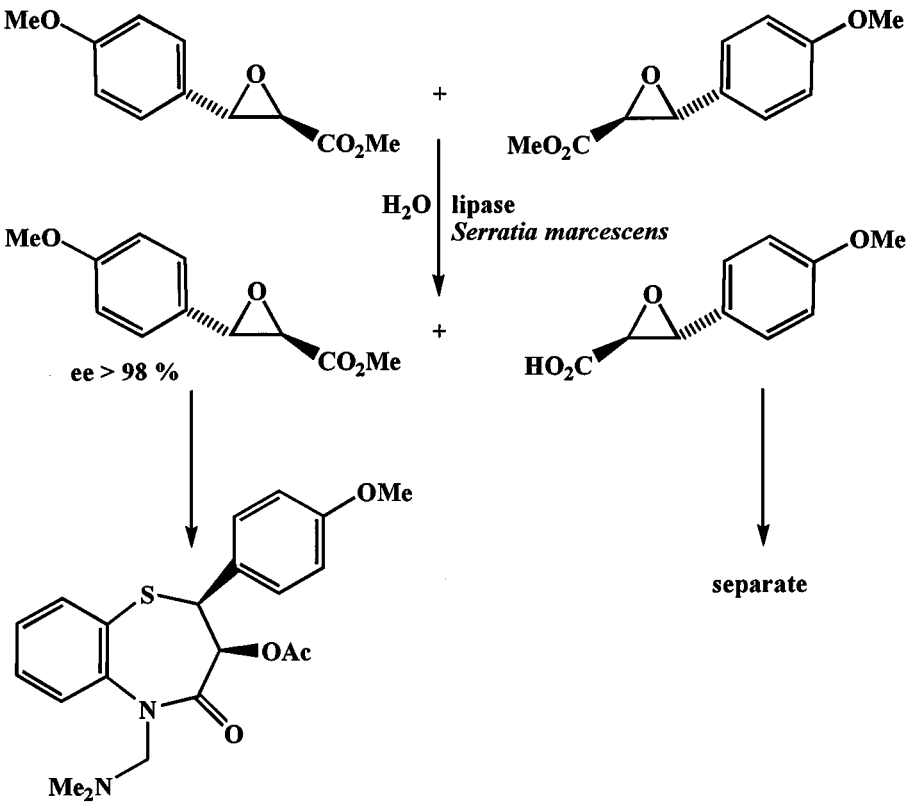

Figure 7 Kinetic resolution of methyl-*p*-methoxyphenylglycidate by *Serratia marcescens* lipase as a key step in the synthesis of Diltiazem.

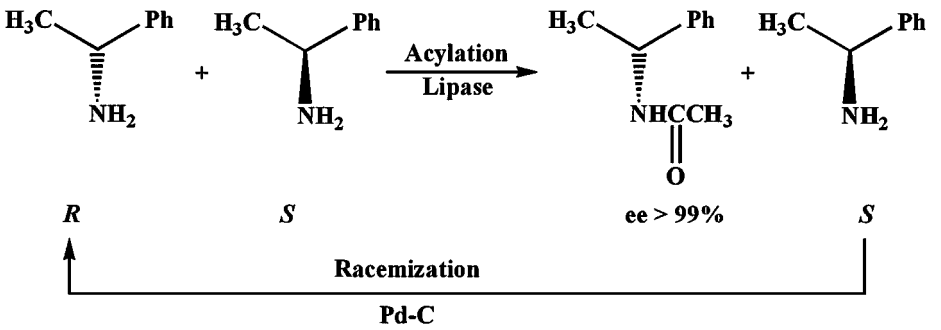

Figure 8 Dynamic kinetic resolution of racemic phenylethyl amine based on enantioselective lipase-catalyzed acylation and racemization by palladium on charcoal (Pd-C).

active amines (8). The lipase from *Burkholderia plantarii* catalyzes the enantioselective acylation of a fairly wide variety of racemic amines (ee > 95%). Perhaps one disadvantage of kinetic resolution is the fact that only 50% of the total material is used. However, methods are starting to be developed that allow for >50% conversion. The underlying principle is "dynamic kinetic resolution" (13, 144, 146). Accordingly, the system not only contains the lipase as an enantioselective biocatalyst but also a second catalyst that causes the rapid racemization of the substrate. Because the product is not racemized under the reaction conditions, 100% conversion to a single enantiomeric product is theoretically possible. This principle opens up completely new perspectives for biocatalysis and indeed for synthetic organic chemistry. In practice, this is difficult to achieve. One stringent requirement is the compatibility of the enzyme with the second catalyst. Nevertheless, several interesting publications illustrating the principle have appeared recently (13, 144, 146). An example is the dynamic resolution of racemic phenylethyl amine based on *N*-acylation catalyzed by the lipase and racemization catalyzed by palladium on charcoal (Pd-C) (116) (Figure 8). Essentially complete enantioselectivity was observed at a conversion of 70%, the side product (30%) being acetophenone.

Immobilization Techniques

It has been claimed that the success of a lipase-catalyzed enantioselective preparation of a certain pharmaceutical depends on immobilization and recyclization of the biocatalyst (20). It is likely that this statement is general. Indeed, in the above mentioned preparation of the chiral intermediate used in the industrial synthesis of Diltiazem, the lipase from *S. marcescens* was supported in a spongy matrix, which was used in a two-phase membrane bioreactor. Other forms of immobilization of lipases have also been described (114). A recent example of a highly efficient technique is based on encapsulation of lipases in hydrophobic sol-gel materials (35a, 112). Accordingly, mixtures of $Si(OCH_3)_4$ and liphophilic alkyl-derivatives $RSi(OCH_3)_3$ are hydrolyzed in the presence of a lipase. The alkyl-modified silica

gel, which is produced in the sol-gel process, grows around the enzyme, leading to encapsulation. Such materials display dramatically increased enzyme stability and activity. In acylation and transesterification reactions, lipase activities of 500–10,000% relative to the use of a traditional nonimmobilized enzyme powders in organic solvents are typical. In addition to separation and recyclization by simple filtration, magnetic separation is also possible, provided that nanoparticles of iron oxide are included in the encapsulation (118). Although the reasons for the increased enzyme activities have not been unambiguously elucidated, it is clear that the sol-gel materials need to contain hydrophobic alkyl groups. In the absence of such hydrophobic groups in the matrix as a consequence of using $Si(OCH_3)_4$ as the sol-gel precursor, $<5\%$ activity is observed. Thus, a type of interfacial activation may be simulated in the alkyl-modified microenvironment of the lipase, although this needs to be proven.

Directed Evolution of Enantioselective Lipases

Although many different compounds are amenable to lipase-catalyzed enantioselective synthesis, limitations obviously exist owing to substrate specificity. If a given substrate shows an unacceptable level of enantioselectivity, it may help to vary the conditions of the reaction (e.g. solvent, temperature). Nevertheless, such empirical attempts cannot be viewed as a general method.

A recent novel approach towards increasing the enantioselectivity of a lipase-catalyzed organic transformation is based on directed evolution. This technique was used previously to develop enzymes with improved stability and activity (5, 115, 154). Mutations are introduced into genes encoding proteins of interest as catalysts by error-prone PCR or recombinative methods such as DNA shuffling. After expression of these mutated genes in suitable microbial hosts, the production of functional biocatalysts results. Selection or simple screening procedures are then used to identify the "best" mutant enzyme in a large library of potential candidates, and the procedure is repeated *n* times (*n* is the number of mutational steps) until the desired catalytic features have been attained. In principle, it should be possible to apply the relevant techniques of molecular biology to create superior biocatalysts having increased degrees of enantioselectivity. However, enantioselectivity is not a simple parameter to deal with. Indeed, several problems need to be addressed, including the all important issue of developing rapid assay systems capable of identifying large numbers of enantioselective biocatalysts.

In a classic paper concerning the creation of enantioselective enzymes by directed evolution, the lipase-catalyzed enantioselective hydrolysis of racemic *p*-nitrophenyl-2-methyldecanoate was chosen as the test reaction (117) [Figure 9*a*]. The bacterial lipase from *P. aeruginosa* PA01, an enzyme composed of 285 amino acids, was used as the catalyst, the wild-type showing an enantioselectivity of only 2% ee in favor of the (*S*)-configured 2-methyldecanoic acid. This means that the lipase has essentially no preference for either of the enantiomeric forms of the racemic ester.

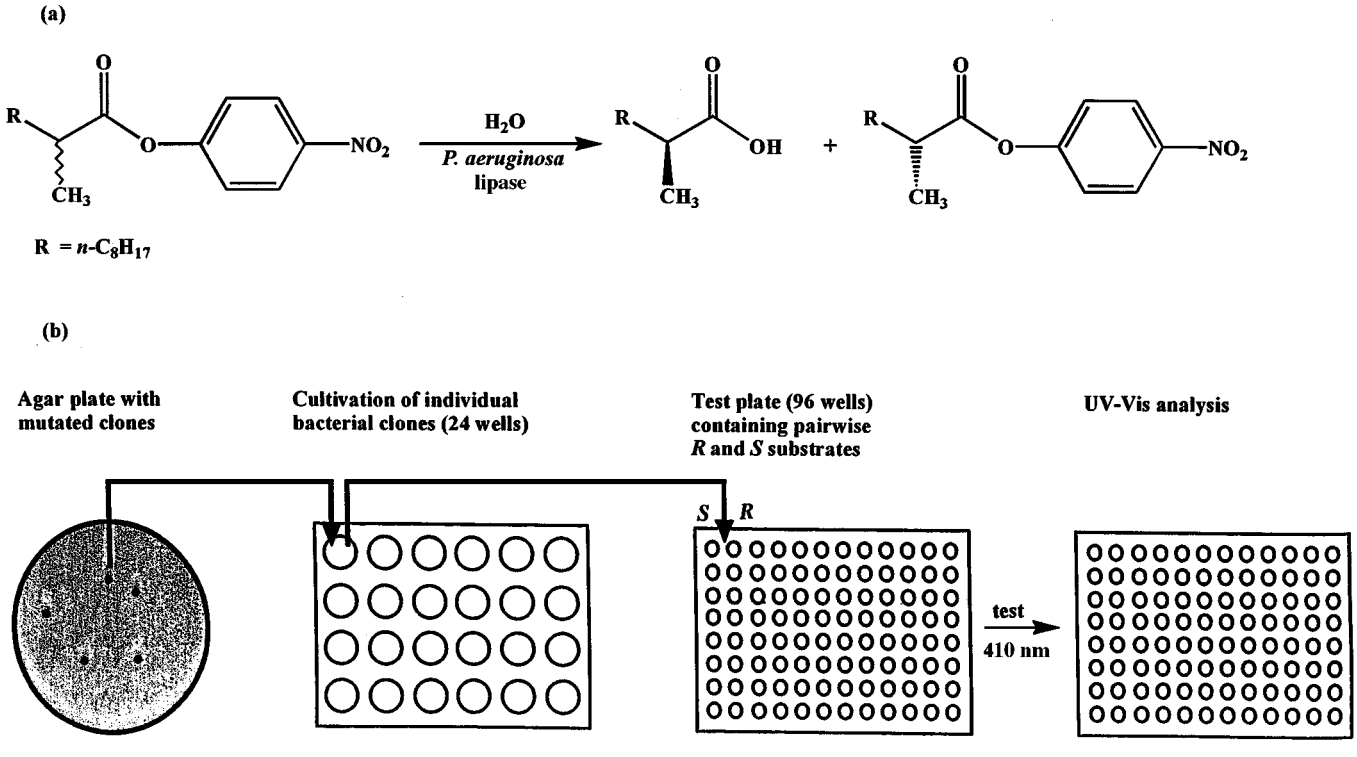

Figure 9 Creation of an enantioselective lipase by directed evolution. (*a*) Kinetic resolution of *p*-nitrophenyl-2-methyldecanoate used as the test reaction. (*b*) Principle of the screening system.

The minimal structural change that mutagenesis could bring about would be the substitution of a single amino acid somewhere along the 285-member amino acid chain. Because the optimal position and type of amino acid substitution cannot be predicted, random mutagenesis was chosen in hope of obtaining a library of mutant lipases in which at least a few members show enhanced enantioselectivity in the test reaction (117). The size of such a library can be calculated to be 5415. Experimentally, the strategy we choose was a low mutagenesis frequency, expecting that a substantial increase in enantioselectivity would result in each generation. By using the error-prone PCR (ep PCR), the lipase gene consisting of 933 bp was subjected to random mutagenesis. The mutated genes were ligated into a suitable expression vector, amplified in *E. coli*, and expressed in *P. aeruginosa*. In the first round of mutagenesis, $\sim$1000 mutants were isolated.

The difficult problem of rapidly screening these for enantioselectivity in the test reaction was solved in the following manner (117). The 96 wells of commercially available microtiter plates were loaded pairwise, with the enantiomerically pure (*R*)- and (*S*)-substrate dissolved separately in dimethylformamide together with the culture supernatants of the respective lipase mutants in Tris/HCl buffer. This means that $\leq$48 mutants/microtiter plate could be screened by measuring the absorption of the *p*-nitrophenolate anion at 410 nm as a function of time (8–10 min). Obviously, if the slopes of the recorded lines of a given pair of reactions are identical, then the enzyme does not distinguish between the two enantiomeric forms of the substrate. Conversely, if the slopes differ considerably, then a certain degree of enantioselectivity must be operating. Figure 9*b* summarizes the principle of the screening system.

Of the 1000 mutants of the first generation tested, about 12 showed enhanced enantioselectivity. The exact values were determined by hydrolyzing the racemic ester in the presence of the corresponding mutant lipases and analyzing the reaction products by gas chromatography on chirally modified capillary columns. The most selective mutant showed an ee value of 31% in favor of the (*S*)-acid. Subsequent mutagenesis experiments gave rise to improved mutants in the second generation (57% ee), third generation (75% ee), and fourth generation (81% ee). The latter corresponds to an enantiomeric product ratio of better than 90:10 and an E value of 11.3 (117). A further increase in enantioselectivity can be expected by applying the following strategies: (*a*) more extensive screening of larger mutant libraries; (*b*) further generations of mutants; (*c*) application of recombinative methods like DNA shuffling; and (*d*) application of cassette mutagenesis. Indeed, initial experiments along these lines, especially the combination of mutagenesis types, have led to the creation of mutant lipases showing ee values of $>$90%. The results clearly show that directed evolution is a rational and viable way to obtain engineered lipases showing dramatically enhanced enantioselectivity in a given reaction. One of the prime virtues is the fact that no knowledge of the three-dimensional structure of the enzyme nor of the reaction mechanism is necessary. In contrast to site-specific mutagenesis, there is also no need to develop intuition or theoretical models as to the position or type of amino acid exchange. Thus, in a certain sense the method

based on directed evolution is strictly rational. Work is in progress to see how general this novel approach to enantioselectivity is. This includes the development of new screening systems for the high throughput evaluation of enantioselectivity. Such research is necessary because the system described here applies only to chiral esters. One approach is based on black body radiation using an appropriate IR camera. Indeed, enantioselectivity of the lipase-catalyzed acylation of a chiral alcohol has been detected by this technique (113). Nevertheless, other approaches to assay enantioselectivity need to be explored, including the possible use of phage display in selection processes.

CONCLUSIONS AND FUTURE DIRECTIONS

The world of bacterial lipases is rapidly expanding. An impressive number of lipase genes have been identified, and many lipase proteins were biochemically characterized. Because lipase reactions take place at an interface, the quality of which greatly influences the obtained results, it is still difficult to compare directly specific enzyme activities as well as regioselectivities or stereoselectivities determined in different laboratories. Although lipases obtained from selected *Pseudomonas*, *Staphylococcus*, and *Bacillus* species can already be produced at an industrial scale, there is still a lack of knowledge concerning regulation and, in particular, mechanisms governing folding and secretion of lipases. The existing three-dimensional structures of lipases allow the identification of domains and amino acid residues involved in substrate binding, catalysis, and enantiopreference, thereby enabling researchers to tailor lipases for selected applications by using site-directed mutagenesis. Because this approach is limited to only a few specific cases, the creation of lipases with novel properties by directed evolution constitutes a more general approach. Indeed, it is likely that this method will become increasingly important. Undoubtedly, there is a steadily increasing demand to identify, characterize, and produce lipases for a variety of biotechnological applications, with special emphasis on enantioselective biotransformations. Therefore, as a first step, standard assay systems should be developed, allowing one to test hydrolysis and synthesis reactions catalyzed by a given lipase. Furthermore, at least one system that allows for heterologous expression and secretion of different lipases needs to be developed. Finally, a data bank should be built comprising sequences of available genes and proteins, a description of their biochemical properties, including specific activities and stereoselectivities, and options available to express and produce these lipases.

ACKNOWLEDGMENTS

Lipase research in the lab of KEJ is continuously supported by the European Commission in the framework of the programs BRIDGE (BIOT-CT-91-0272) and BIOTECH (BIOT-CT91-272 and BIO4-CT98-0249). The authors thank Susanne

Tetling for preparation of Figures 1 and 2, Dietmar Lang for providing Figure 4, and Uli Winkler for critical reading of the manuscript.

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